PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Gasco et al.	Confirmation No.:	4539
Serial No.:	10/533,512	Art Unit:	1612
Filed:	February 5, 2005	Examiner:	HUANG, Gigi G.
Title:	"Pharmaceutical compositions suitable for the treatment of ophthalmic diseases"		

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.132

I, Maria Rosa Gasco, being duly sworn depose and say that:

1. I am an Italian citizen residing at: Torino (IT)
2. I am familiar with the English language.
3. I graduated in: Chemistry (1953) and in Pharmacy (1973) at the University of Turin
4. I am co-inventor of the above-identified US patent application.
5. I am the President and Scientific Director of Nanovector S.r.l., an Italian company (located in Turin, IT) focused on pharmaceutical nanotechnologies.
6. I am also a senior academic Professor of Technology, Socioeconomics, and Pharmaceutical law at the University of Turin.
7. I am author of more than 140 scientific publications and I am inventor of more than 60 patents/patent applications.

Experimental section

In order to further support the patentability of the claimed invention, I herewith enclose and herein below comment two studies, where once again it has been demonstrated the efficacy of the claimed method.

Particularly, said studies are:

1) Program#Poster#:4464/D630
Gargini M. C. et al., "Inhibition of Ceramide de novo Synthesis in an Animal Model of Retinitis Pigmentosa: II. Effects on Photoreceptor Survival and Function", ARVO 2009 annual meeting, pp 50, D629--, Fort Lauderdale, 2009;

2) Program#Poster#:4463/D629
Ghidoni R. et al., "Inhibition of Ceramide de novo Biosynthesis in an Animal Model of Retinitis Pigmentosa: I. Morphological and Biochemical Effects", ARVO 2009 annual meeting, D630--, Fort Lauderdale, 2009.

Said studies demonstrated in a mammalian model of Retinitis Pigmentosa (RP) that it is possible to decrease the rate of apoptotic death of photoreceptors by lowering retinal ceramide levels through inhibition of the de novo biosynthesis of this molecule. Non invasive, chronic administrations of solid lipid nanoparticles according to the present invention loaded with SPT inhibitors are effective in increasing the survival rate and functional responses of photoreceptors. Since typical RP has a naturally slow evolution, a further delay in the degeneration of photoreceptors might be considered itself therapeutic. Particularly, it is known that a small increase in the survival of rods produces a proportionally larger increase in the survival rate of cones, the only cells upon which residual vision is based in RP patients.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: April 26, 2010

Maria Rosa Gasco

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Presentation Abstract

Program#/Poster#:	4463/D629
Abstract Title:	**Inhibition of Ceramide *de novo* Synthesis in an Animal Model of Retinitis Pigmentosa: II. Effects on Photoreceptor Survival and Function**
Presentation Start/End Time:	Wednesday, May 06, 2009, 1:45 PM - 3:30 PM
Location:	Hall B/C
Reviewing Code:	331 photoreceptor degeneration/apoptosis: cell biology and pathology - RC
Author Block:	*M. Gargini[1], A. Asta[1], I. Piano[1], P. Gasco[2], C. Musicanti[2], E. Novelli[3], E. Strettoi[4], R. Ghidoni[5].* [1]Psychiatry & Neurobiology, University of Pisa, Pisa, Italy; [2]Nanovector srl, Turin, Italy; [3]Bietti Foundation, Rome, Italy; [4]Neuroscience Institute CNR, Pisa, Italy; [5]DIMCO, San Paolo Medical School, Milan, Italy.
Keywords:	511 electroretinography: non-clinical, 427 apoptosis/cell death, 737 transgenics/knock-outs

Abstract Body:

Purpose: In the companion paper, we show that ceramide biosynthesis plays a role in the apoptotic pathway leading to photoreceptor death in a mouse model of Retinitis Pigmentosa (RP). Here, we attempt to slow down photoreceptor degeneration in the same mutant by chronic administration of inhibitors of serine palmitoyltransferase (SPT), the enzyme that catalyzes the limiting step of ceramide biosynthesis. We use a nanotechnology- based preparation to deliver drugs to the retina of rd10 mutant mice, later assessing retinal preservation and functional performance by means of histology and ERG recordings.

Methods: Mice of the *rd10* strain were used. These carry a missense mutation of the rod-specific phosphodiesterase leading to a degeneration of photoreceptors from P14 rd10 animals aged P14 to P35 were administered daily eye drop formulation of SPT inhibitors loaded into Solid Lipid Nanoparticles (SLN) obtained from warm microemulsion (Nanovector srl, Italy). Control mice were given unloaded lipid particles. Scotopic and photopic ERG recordings were obtained from animals of various ages (P20; P25; P30; P35). Retinal sections from ERG recorded animals were stained with nuclear dyes and examined at a confocal microscope for measurements of the photoreceptor layer thickness.

Results: Chronic administration of SPT inhibitors and of unloaded SLN formulation did not cause adverse local or general effects. ERG responses could be evoked from treated rd10 mice even after complete ERG extinction in untreated, control animals. Scotopic a-waves were recordable from untreated mice only in the P20-P22 window but persisted clearly even after P30 in treated littermates. Histological examination of treated and untreated retinas at P24 (the peak of rod apoptosis in this strain) showed prolonged survival of photoreceptors with a higher number of nuclei rows in the outer nuclear layer.

Conclusions: Non invasive, chronic administrations of nanoparticle loaded SPT inhibitors are effective in slowing down photoreceptor death and preserving the

ERG response in a known mouse model of RP. This is particularly relevant in view of the fact that typical RP has a naturally slow evolution; a strategic way to attack this disease is to further delay the degeneration of photoreceptors, and particularly the secondary death of cones, the only cells upon which residual vision is based in RP patients.

Commercial Relationships: **M. Gargini**, Inventor, P; **A. Asta**, None; **I. Piano**, None; **P. Gasco**, Nanovector srl, Turin, Italy, E; Nanovector srl, Turin, Italy, P; Nanovector srl, Turin, Italy, R; **C. Musicanti**, Nanovector srl Turin, Italy, E; **E. Novelli**, None; **E. Strettoi**, Inventor, P; **R. Ghidoni**, Inventor, P.

Support: MIUR, PRIN 2007 (MCG) ; BRPS (ES); R01 EY 12654 (ES)

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Presentation Abstract

Program#/Poster#:	4464/D630
Abstract Title:	**Inhibition of Ceramide *de novo* Biosynthesis in an Animal Model of Retinitis Pigmentosa: I. Morphological and Biochemical Effects**
Presentation Start/End Time:	Wednesday, May 06, 2009, 1:45 PM - 3:30 PM
Location:	Hall B/C
Reviewing Code:	331 photoreceptor degeneration/apoptosis: cell biology and pathology - RC

Author Block: *R. Ghidoni[1], G. Sala[1], P. Signorelli[1], E. Novelli[2], B. Ilaria[3], M. Gargini[4], E. Strettoi[3].* [1]Medicine, Surgery and Dentistry, University of Milan, Milano, Italy; [2]Bietti Foundation, Rome, Italy; [3]Neuroscience Institute, CNR, Pisa, Italy; [4]Psychiatry & Neurobiology, University of Pisa, Pisa, Italy.

Keywords: 427 apoptosis/cell death, 645 photoreceptors, 692 retinal degenerations: cell biology

Abstract Body: **Purpose:** In *Retinitis Pigmentosa* (RP) photoreceptor death occurs by apoptosis but the individual pathways of this process are unknown. We tested two hypotheses: 1) that the sphingolipid ceramide plays a pro-apoptotic role in the degeneration of photoreceptors in an animal model of RP; 2) that the *in vivo* administration of inhibitors of *de novo* ceramide biosynthesis decreases photoreceptor death.
Methods: *rd10* mutant mice were used. These carry a missense mutation of the rod-specific phosphodiesterase leading to photoreceptor death from P14. *rd10* and *wt,* control mice, aged P14, P22, P30 and P37, were anesthetized, their retinas isolated and frozen on dry ice. For ceramide quantification, the two retinas of each animal were washed, pooled, and endogenous ceramide content determined by the diacylglycerol kinase assay. Ceramide values of different animals were averaged and referred to total phospholipids.
Intravitreal injections of non toxic amounts of commercially available inhibitors of serine palmitoyltransferase (SPT, the rate-limiting enzyme of ceramide biosynthesis), were performed in the right eyes of *rd10* mice at P19. Left eyes were injected with vehicle. Treated and control eyes were isolated 48 hrs after injection and used for biochemical assays of ceramide or retinal histology. Photoreceptor degeneration rates were assessed by counting pyknotic (apoptotic) nuclei in the outer nuclear layer of whole mounted retinas after fluorescent nuclear staining and confocal microscopy.
Results: Retinal ceramide levels in rd10 mice double from P14 to P30, the time interval of maximum photoreceptor death. Endogenous ceramide content of *wt,* normal mice, is lower than that measured in *rd10* retinas at the peak of apoptosis. Intraocular injections of SPT inhibitors have no toxic effects at local or general levels but reduce significantly retinal ceramide levels in *rd10* compared to control retinas. Intraocular treatment with SPT inhibitors decrease the number of pycnotic photoreceptors in rd10 mutant mice by approximately 50%.
Conclusions: This study demonstrates for the first time in a mammalian model of

RP that it is possible to decrease the rate of apoptotic death of photoreceptors by lowering retinal ceramide levels through inhibition of the *de novo* biosynthesis of this molecule. This approach can be regarded as a therapeutic strategy to maintain photoreceptors (otherwise doomed to death) viable in perspective of a subsequent RP treatment.

Commercial Relationships: **R. Ghidoni**, Inventor, P; **G. Sala**, None; **P. Signorelli**, None; **E. Novelli**, None; **B. Ilaria**, None; **M. Gargini**, Inventor, P; **E. Strettoi**, Inventor, P.

Support: MIUR: PRIN 2007 (MCG); BRPS (ES); R01 EY 12654 (ES)